GENETIC ANALYSIS OF DILATED CARDIOMYOPATHY IN THE GREAT DANE

A Dissertation

by

STEPHANIE MICHELLE HERBST

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Genetics

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Approved by:

Chair of Committee,	Keith E. Murphy
Committee Members,	Ann B. Kier
	Charles R. Long
	Theresa W. Fossum
Chair of Genetics Faculty,	James R. Wild

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ABSTRACT

Genetic Analysis of Dilated Cardiomyopathy in the Great Dane. (December 2007)

Stephanie Michelle Herbst, B.S.; B.S., Texas A&M University

Chair of Advisory Committee: Dr. Keith E. Murphy

The domestic dog, *Canis familiaris*, with over 450 naturally-occurring hereditary diseases, serves as a valuable model organism for study of the genetics underlying many human hereditary diseases. Approximately half of the diseases that afflict the dog are clinically very similar to various human hereditary diseases. Several cardiac diseases are in this category. Our laboratory is interested in cardiac diseases because they are common causes of death in the human and are also a widespread problem in many breeds of dog. The specific focus of my work is on understanding the genetics of dilated cardiomyopathy (DCM).

DCM is a disease characterized by enlargement of the left ventricle leading to an inability of the heart to pump sufficient blood to the body. It is one of the most common cardiac diseases in the dog and has a high mortality. The Great Dane is the second most commonly affected breed. It is seen in many families of Great Danes, and this suggests that DCM has a genetic component. The mode of inheritance of DCM in the Great Dane is currently unknown, although studies have reported both autosomal recessive and autosomal dominant transmission.

Many different genes cause DCM, indicating the complexity of the disease. These typically produce proteins that are involved in the sarcomere or cytoskeletal components, leading to problems with contraction or cardiac cell integrity.

In order to identify causative or susceptibility genes for DCM in the Great Dane, a whole-genome linkage screen was conducted in a family of Great Danes. One candidate gene, *gamma-sarcoglycan* (*SGCG*), was identified through linkage and sequenced in affected and unaffected dogs. Sequencing data revealed no mutations in the coding regions of *SGCG*, most likely excluding it as a candidate gene for DCM. Continued evaluation of this gene and others, both in sequence content and additional properties such as epigenetic effects, protein structure, and interaction with other genes will increase understanding of DCM in both the dog and the human.

DEDICATION

For Todd, Madeleine, and Anna:

You are everything

ACKNOWLEDGEMENTS

I would like to first thank my advisor, Dr. Keith Murphy, for allowing me to be a part of the Texas A&M University Canine Genetics Laboratory and for his absolute dedication to canine genetics research. My other committee members, Dr. Theresa Fossum, Dr. Charles Long, and Dr. Ann Kier, were also invaluable in my graduate education, and they helped me throughout the duration of my research.

The Great Dane Club of America and the Great Dane Health Foundation of America made this research possible with donation of samples and support, and they deserve special recognition, as do all of the owners and breeders involved with this project. Their tireless efforts in recruiting, collecting information, and simply recognizing this research was very helpful and I could not have done any of this without them.

I would also like to thank the members of the Canine Genetics Laboratory for providing me with constant help and support. In particular, I would like to recognize Jacquelyn Wahl, Dr. Ashley Hamlett, Jessica Moody, and Michelle Boggs. Their support and great friendship have made such a difference to me, and I value them a great deal.

Finally, I wish to thank my family. My parents, Richard and April Leech, have provided me with support in every area when I needed it. In particular, my husband, Todd, and my two daughters, Madeleine and Anna, have played the greatest role in my success. They are my support, my laughter, and my life. Without them, I would not be at the point at which I am today. Thank you.

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CHAPTER I

INTRODUCTION

Evolution of the dog

The domestic dog, *Canis familiaris*, has origins in East Asia, where it diverged exclusively from the gray wolf[1-3]. The exact point at which this divergence occurred, however, has proven difficult to establish. The modern dog, consisting of over 1000 different recognized breeds throughout the world[4,5], has developed within the last several hundred years and was, in fact, the first animal to be domesticated[6,7]. However, there are different estimates of the age of the dog in general, and even whether its relationship with humans occurred as a single event or was the result of multiple individual domestication events[8]. Because the dog exhibits more genetic diversity than would be expected from a single domestication event such as a genetic bottleneck, it is possible that multiple backcross events, such as that between a more domestic dog and a non-domesticated relative, occurred during the domestication process [7,8]. Furthermore, the striking level of phenotypic diversity found in the dog suggests pronounced genetic variability of its ancestors, which indicates a more diverse origin[9].

Wayne and Vilà compared mitochondrial DNA sequence variation between wolves and dogs and suggested that the modern dog came into existence approximately 135,000 years ago[1,10,11]. Savolainen argued for a more recent domestication that occurred approximately 25,000 years ago resulting in the dog as it is known today[1,10]. In contrast

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This dissertation follows the style of Journal of Veterinary Cardiology.

to mitochondrial DNA evidence, however, the earliest accepted fossils of modern dogs found in Germany, Israel, and Iraq date to 12-14,000 years ago[3].

Canis familiaris as a model organism

Since its domestication, the dog has played a number of roles in the lives of humans. It has an important role in biomedical research and is particularly useful for genetic studies. The dog is widely known, both currently and historically, to be man's companion and, as such, shares much of the same environment as humans, including living quarters and food sources. This allows the dog to be evaluated in the same context as the human and eliminates the need to control for environmental differences between the two. In addition, since the advent of breed clubs and registries such as the American Kennel Club (AKC), it is easy to assemble information such as breed records and multigenerational pedigrees[12]. Breed clubs exist, in part, to ensure that breed standards are maintained. This is exemplified by the "breed barrier" rule, a requirement of the AKC that both parents of a particular dog be registered within the same breed[12]. Thus, each breed represents a closed breeding population. Because of this, the domestic dog has become an important model organism for the study of hereditary diseases.

There is a level of phenotypic diversity in the dog that is not found in any other mammal[5,11]. Selective breeding practices maintain phenotypic characteristics of individual breeds, including size, coat color and texture, and body shape. Furthermore, the strict breed standards ensure reproductive isolation between breeds[12]. This closed gene pool between breeds allows each breed of dog to be studied as individual isolated

populations[12,13]. Due to extensive inbreeding, the popular sire effect, population bottlenecks and founder effects, more than half of the hereditary diseases that afflict the domestic dog are restricted to one or only a few breeds[2,13].

The breeding practices necessary to maintain breed standards have resulted in the selection for hereditary diseases in the dog, of which there are more than 450[14], with new diseases reported every year[15]. Most of the diseases are inherited in an autosomal recessive fashion[16], and become more prevalent when the high levels of inbreeding seen in most breeds of dog produce more affected dogs as a result of two asymptomatic dogs carrying the recessive alleles being bred together. About half of the hereditary diseases found in the dog are represented in the human, as well, with clinical similarities between the two[14]. Therefore, the dog serves as an excellent natural model of human hereditary diseases, eliminating the need for induced models, such as transgenic models made in the mouse or rat, which have historically been the most popular model systems in which to study human diseases. In fact, sequence similarity between the human and dog is at least twice that found between the mouse and either the human or the dog[17,18]. Thus, using the dog as a model system provides the opportunity to more fully evaluate hereditary diseases in humans, because the dog is evolutionarily closer to the human than is the mouse. In addition, drug safety and gene therapy trials have also successfully demonstrated the utility of the domestic dog in areas other than hereditary diseases[17].

The dog has several additional qualifications that also make it a useful model system. The medical care available for the domestic dog is second only to that available

for the human[13,15,17]. In addition, dogs are able to be crossbred easily, and have short gestation times and large litter sizes relative to the human.

Resources available for canine genetics research

The canine genome consists of approximately 2.5 billion base pairs[2], which is slightly smaller than the size of the human genome (2.9 billion base pairs). The estimated number of genes is similar for both, approximately 20-25,000. The dog has 38 pairs of acrocentric chromosomes and metacentric sex chromosomes, for a total of 78 chromosomes. Many of the canine chromosomes are very similar in size and morphology, with only slight differences in size, making their distinction difficult[19]. The first 21 chromosomes, as well as the X and Y chromosomes, were identified in 1996 by Switonski, *et al*., with the use of G-banding techniques[20], while the remaining chromosomes were not definitively identified until 1999 by Breen, *et al*., with DAPI banding of chromosome-specific paints[21].

The proven utility of the dog for study of genetics, and specifically as a model for human hereditary diseases[14], resulted in its selection for genomic sequencing. The first draft of the sequence of the canine genome was completed in 2002 by Kirkness, *et al*. of a male standard poodle[22]. It consisted of 6.22 million sequence reads and provided 1.5x coverage of the canine genome. Even though this sequence effort represented only approximately 78% of the canine genome, it was determined that there is significant nucleotide conservation between the dog and other mammalian genomes, specifically, more so between the dog and the human than between the human and the mouse[10]. In

fact, the 1.5x sequence of the dog was found to annotate more human genes than the 8x murine genome sequence[17]. The sequence also revealed that the canine genome consisted of approximately 31% repetitive sequences such as microsatellite DNA and SINE sequences, which are useful in mapping of disease genes[10].

The presence of large gaps in the genome sequence of the poodle led to a second sequencing effort of the canine genome, which was completed in 2004. A female Boxer was selected for sequencing after determining that she exhibited the smallest amount of genetic heterogeneity and would, therefore, best represent the canine genome in general[23,24]. This sequencing effort produced a total of 31.5 million sequence reads for sequence coverage of 7.5x and is publicly available online at the website of the National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/ [2]. This is estimated to cover approximately 99% of the genome, with approximately 1% of the genome remaining as gaps in sequence. These sequence data suggested that the coding portions of the genome of the dog are approximately 18% smaller than the coding regions of the human and only 6% smaller than that of the mouse. This difference is likely due to the lower rate of insertions in the canine genome relative to both the human and mouse[25].

In addition to the genome sequence, the generation of dense marker maps has expedited the search for disease genes. Linkage maps have progressed from the first generation linkage map, which included 43 microsatellites placed into 16 linkage groups[26], to the current best tool for conducting linkage studies, the MSS-3, which is a set of 510 microsatellite markers evenly spaced throughout the canine genome. The

MSS3 allows genome screens in the dog to be conducted at a resolution of approximately 5 cM[27].

Methods for finding disease genes

There are three common methods that are used in studies of hereditary diseases. First, the candidate gene approach is used when a causative gene is known in another species. The orthologous gene is located in the dog and analyzed for causative mutations. This method is well-suited for comparative studies of hereditary diseases in the dog, because the analytical tools are now available and widely used. This method has successfully been used to find the causative gene for many canine diseases. A disadvantage of the candidate gene approach is the significant investment of time and resources that often result in negative results, *i.e.* exclusion of genes[28]. For this reason, the candidate gene approach is feasible for diseases that have few possible genes, but less useful for complex, polygenic diseases, due to the large numbers of genes that must be analyzed.

Linkage is the study of coinheritance of alleles. Genes that are located near one another tend to be inherited together as a haplotype. This can be exploited in the search for disease genes because microsatellite sequences that are coinherited with a disease gene can identify regions in the genome that are linked to a disease phenotype and therefore potentially harbor causative mutations. There are two types of linkage analyses that can be conducted, classical linkage studies and linkage disequilibrium, or association studies. The purpose of these studies is to identify genomic loci that are

involved in a trait without any assumptions as to the genetic mechanism at work for the trait[29]. Because loci, and not particular genes, are identified, fine mapping can be done to further narrow the region of interest. Classical linkage utilizes a pedigree that consists of informative breedings so as to enable the study of transmission of the various alleles of a marker. For studies of hereditary diseases, this requires a multigenerational pedigree of dogs, both affected and unaffected, and the ability to analyze transmission of alleles so as to be able to assess differences between affected and unaffected individuals. Furthermore, classical linkage analysis assumes that the mutation causative for the disease occurred as a founder event in the population or breed. Also, it is assumed that a finite number of recombination events have occurred in the pedigree in order to increase the chances of finding linkage. This approach resulted in identification of the genes for several monogenic hereditary diseases, including cystic fibrosis and Huntington's disease[29]. Genetically heterogeneous traits, on the other hand, may not be well-suited for linkage studies, because it is difficult to determine mode of inheritance when there is more than one contributing locus.

Linkage disequilibrium (LD) is the method of choice when the population consists of random, unrelated dogs that are separated by a minimum of three generations. This method is often employed in studies of the dog due to the difficulty in obtaining enough related dogs from a pedigree for studies of a particular disease. Due to the random nature of the participants in an LD study, it is assumed that there are a potentially unlimited number of recombination events that have occurred since the first appearance of the disease in the population. This unlimited number of recombination

events essentially “breaks” the genome into smaller and smaller pieces, reducing the size of the haplotype blocks that are inherited from generation to generation. This increases the likelihood of finding linkage to a disease gene, especially because LD also assumes that the mutation occurred as a founder event in the population or breed. A disease-causing mutation that occurred many generations ago and lies in close proximity to a genetic marker will most often be coinherited with that marker, leading to a population-wide association. This phenomenon is easily explained by the fact that the population has a shared common ancestry[30]. In essence, if all dogs in a breed that are affected with the same hereditary disease harbor the same mutation, the value for LD will be higher. In short, if a genotype is truly linked to a disease in a population, it will be found more often in affected individuals than in unaffected individuals. LD analysis, however, is a more difficult method to employ in the study of rare, complex diseases, because of the requirement of a large number of affected individuals, which is often not possible to obtain.

A third approach to identification of potential disease-causing genes is microarray analysis. Probing of microarrays is done in order to measure mRNA levels. This method has been used to identify candidate genes for hereditary diseases of dogs. Microarray analyses have typically been performed on cDNA oligonucleotide arrays to detect homology between transcripts. However, it is now possible to perform analyses on arrays containing tens of thousands of single nucleotide polymorphisms (SNPs), a method which will, in theory, combine both linkage studies and microarray studies.

These SNP arrays consist of a very large number of SNPs that are present in the canine genome and can be used for linkage studies of hereditary diseases.

Dilated cardiomyopathy

A common cause of death for humans and dogs is cardiac disease. One such cardiac disease is DCM. DCM is the most common type of cardiomyopathy, and accounts for about 60% of all human cases[31]. DCM was first described in 1970 by Ettinger, Bolton, and Lord as congestive heart failure (CHF) with dilation of the heart in the absence of other cardiac disease[32]. In humans, DCM is the leading reason for cardiac transplant[33]. DCM is a chronic progressive disease of the cardiac muscle that causes enlargement of the left, and sometimes right, ventricle[34]. This enlargement impairs the systolic function of the heart and results in the inability of the heart to pump sufficient blood to the body. DCM affects several large breeds of the domestic dog, including the Doberman pinscher, Great Dane, Boxer, Portuguese water dog, and Irish Wolfhound[35]. The Great Dane has the second highest incidence of DCM and is the main focus of this dissertation.

DCM is a heterogeneous disease, in both clinical manifestation and genetic causes[36]. Typically, DCM is an adult-onset disease in the dog. However, age of onset and possible contribution from environmental factors make it more difficult to identify affected individuals[28]. Additionally, affected individuals often reproduce before being identified, thereby propagating the disease. There are childhood onset forms of DCM in the human, but the Portuguese water dog is among the few breeds reported to have early-

onset forms of DCM[37]. Symptoms can include coughing, shortness of breath, exercise intolerance, syncope, cardiac arrhythmias, specifically atrial fibrillation, premature ventricular contractions (PVCs), or even sudden death.

There are two commonly recognized stages of DCM. Preclinical DCM is the stage during which cellular changes begin to occur. During this period, dogs do not show clinical signs of DCM. Preclinical DCM may be brief for some dogs, but in others it can last for years. For these latter dogs, normal arterial blood pressure, resistance, blood flow, and oxygen delivery to the tissues are observed[38]. Preclinical DCM is best diagnosed through echocardiography, which assesses systolic and diastolic function and provides estimates of filling pressures for both sides of the heart[39]. The second stage, overt DCM, is when most diagnoses are made. At this point, the typical clinical signs and symptoms of DCM are evident. This stage is also the most difficult to treat, due to extensive reduction in cardiac function. The prognosis for dogs with overt DCM is grim, with survival times ranging from weeks to months. However, early intervention and treatment may halt progression of the disease and prevent sudden death[40]. Dogs with DCM eventually progress to CHF if sudden death does not occur[34].

DCM is characterized by several common clinical features. Diagnosis of DCM requires pronounced dilation of the left, and possibly right, ventricle, compromised systolic function, and increased sphericity of the left ventricle[41]. As the disease begins, cardiac cells cease normal function in order to maintain the pumping efficiency of the heart and tissue perfusion[38,42]. These changes eventually become maladaptive and begin to promote ventricular remodeling, death of cardiac cells, and fibrosis of affected cardiac

tissue. This, in turn, causes a decrease in the systolic and diastolic function and eventually leads to cardiac failure.

Two distinct histopathological findings are common in canine DCM. These are attenuated wavy fibers, and fatty infiltration. The attenuated wavy fiber type of DCM is characterized by thinner myocytes that have a wavy appearance and are separated by clear spaces indicative of fluid. This is typically found in large breed dogs such as the Great Dane and Irish wolfhound and supports the theory of cytoskeletal elements being involved in DCM. Detection of attenuated wavy fibers in the myocardium has been reported to be reliable for detection of DCM[32]. The fatty infiltrative type of DCM is typically found in Boxers and some Doberman pinschers and is characterized by myocytolysis, degeneration of myofibers, and atrophy of myocytes with fibrosis and fatty infiltration that replaces the myofibers[41]. These findings are thought to precede clinical symptoms, and may reflect the earliest stages of DCM [32].

Etiology and inheritance of DCM

DCM is caused by many different factors. These can include, but are not likely limited to, nutritional deficiencies, viral causes, problems with immune function, toxins, including alcohol, and genetic causes[43]. Studies of human DCM show that most cases (47%) are idiopathic[44]. However, familial cases of DCM are also known, and estimated to account for up to 30% of human DCM[31]. Determination of the mode of inheritance of DCM in a population has proven difficult, and is made more difficult by possible

incomplete penetrance, variable expressivity, and modifier genes that either enhance or suppress the function of the causative allele[28].

The most common reported mode of transmission is autosomal dominant. This accounts for 56% of human familial DCM[45]. However, autosomal recessive, X-linked, and mitochondrial modes of inheritance have been reported in the human, as well[44]. In the domestic dog, familial DCM has been reported in the Doberman pinscher, Boxer, and Portuguese water dog[35]. Autosomal recessive inheritance of DCM is uncommon[46] but has been reported in the Portuguese water dog as a juvenile onset form of DCM [47]. X-linked DCM has been reported in the human[36], but convincing evidence of X-linked DCM in the dog is lacking[35,48].

Genes involved in DCM

Prior to the discovery of causative genes, many regions of the human genome were linked to DCM. Traditional linkage studies have identified many loci that are linked to autosomal dominant DCM in humans, including 1p1-1q1[49], 1q32[50], 3p22-p25[33], 10q21-23[31], 9q13-q22[51], and 6q23[52]. X-linked DCM in humans was found to be linked to Xq28[53] and Xp21, which harbors the dystrophin gene. Mutations in the dystrophin gene cause DCM and are, of course, causative for Duchene Muscular Dystrophy[49]. Also, Barth syndrome, is a disease caused by mutations in the Tafazzin (*TAZ*) gene. Tafazzin mutations result in a highly lethal form of cardiomyopathy[46].

The role of mitochondria in DCM has been investigated, due to the extremely high energy requirements of cardiac cells. Mitochondrial mutations have been known to

cause DCM, but typically not without the involvement of other organ systems, as well[49]. Furthermore, while no causal relationships have been established for DCM and mitochondrial mutations, it is thought that SNPs in mitochondria-encoded genes may be associated with a predisposition to non-familial DCM in some human populations[54].

Once different regions were found to be linked to DCM, individual genes causing DCM in particular populations were identified. These are typically determined to be components of the cytoskeleton or sarcomere, although some genes produce DCM through unknown molecular mechanisms. Many different mutations lead to the clinical symptoms of DCM, indicating the level of complexity and genetic heterogeneity of the disease[45]. The list of genes implicated in human DCM is extensive and includes: sarcomere proteins troponin T (*TnT*)[55,56], troponin I (*TnI*), troponin C (*TnC*), cardiac actin[57], β-myosin heavy chain (*β-MHC*), myosin binding protein C (*MyBP-C*), and α-tropomyosin; cytoskeletal proteins desmin[58], δ-sarcoglycan, vinculin, and titin; and nuclear envelope protein lamin A/C[45,59]. Xq28 harbors *G4.5*, which was determined to be the causative gene for Barth syndrome[60]. *G4.5* encodes many different mRNAs that, through differential splicing, produce different proteins called tafazzins. Barth syndrome results when point mutations introduce premature stop codons to produce truncated proteins. The other identified form of X-linked DCM results from mutations in the dystrophin gene, which is a cytoskeletal protein important in cardiac function. Dystrophin acts to maintain connections between the sarcomere and the cytoskeleton, stabilizing the cardiomyocytes.

Discovery of a causative gene or genes for DCM would allow the development of a DNA-based test for the disease. For the domestic dog, this would enable breeders to identify affected and carrier dogs, and to eliminate them from the breeding pool. This could serve to largely reduce or even eliminate the disease in a breed.

CHAPTER II

WHOLE GENOME SCREEN TO DETECT LINKAGE OF DILATED CARDIOMYOPATHY IN A FAMILY OF GREAT DANES

Introduction

Dilated cardiomyopathy (DCM) is a condition observed in the Great Dane, Boxer, Doberman pinscher, Portuguese water dog, and other breeds of *Canis familiaris*, the domestic dog. The Great Dane is the second most commonly affected, with a prevalence of 3.9%[61]. DCM is the most common form of canine primary cardiovascular disease, accounting for approximately 60-87% of all cardiomyopathy cases[62]. Typically, the disorder is characterized by progressive enlargement of the left ventricle and consequential inhibition of the heart's ability to pump blood throughout the body. Over time, chronic disruption of proper blood flow and the associated sub-optimal oxygenation of tissues lead to arrhythmias, coughing, syncope, ascites, and, in more severe cases, congestive heart failure and sudden death[63].

Veterinarians typically recognize two distinct clinical stages of DCM. The *preclinical stage* is highly variable in duration and refers to the etiologic period when signs and symptoms are not noticeable upon routine examination. *Overt DCM* refers to the window of time when clinical signs and symptoms emerge and the debilitating health effects of the disease become readily apparent in affected animals. Typically, diagnoses are made in the overt stage, when dogs are between four and six years of age[64,65].

As one might expect, disease detection and intervention are most beneficial to affected animals during the preclinical stage when palliative medications, designed to maintain the pumping action of the heart and eliminate excess fluid caused by impaired cardiac function[36], have the greatest impact on quality of life and long-term survival. As such, a genetic test for DCM would be valuable, especially in breeds such as the Great Dane in which the disorder imposes considerable physical, emotional, and economic burdens on dogs and their owners. Several studies are attempting to identify causative or susceptibility genes that influence disease onset and severity in different breeds.

The prevalence of DCM in several breeds of the dog, as well as the fact that DCM is notably rare in cross breeds, suggests a genetic basis[66]. While several different patterns of inheritance for DCM have been reported in the dog[36,66,67], the inheritance of DCM in the Great Dane has not yet been determined, although one theory proposes that it is inherited in an X-linked recessive fashion, at least in some families[68]. Furthermore, the complex nature of cardiac diseases, which do not obey traditional Mendelian genetics, means that they are likely to be the result of multiple inherited and environmental factors[30] and complicated by incomplete penetrance and variable expressivity[69].

While analyses in this study did not yield compelling support for Mendelian transmission or significant linkage, positive LOD scores were identified for eight markers spread across six canine chromosomes. One such marker, FH2324, is a microsatellite located proximal to the gamma-sarcoglycan gene (*SGCG*) on chromosome 25. SGCG is a member of the sarcoglycan complex of transmembrane proteins that

associate with dystrophin to connect the cytoskeleton to the extracellular matrix in skeletal and cardiac muscle[70]. This finding prompted sequencing of the coding region of *SGCG* and evaluation of its role as a potential candidate gene for DCM. The sequencing results indicated that *SGCG* does not harbor a causative mutation in our population, and therefore it was excluded as a candidate gene for DCM in the Great Dane.

Animals, materials and methods

Experimental Population

For this study, 25 client-owned Great Danes were evaluated. The dogs are from a family consisting of four generations and six individuals diagnosed with DCM by a board-certified veterinary cardiologist (see Figure 1). All clinical information and biological samples (blood or buccal swab) were collected for each dog following university regulations. Diagnoses of DCM were based on echocardiography, the AVMA-approved diagnostic criterion for DCM[64].

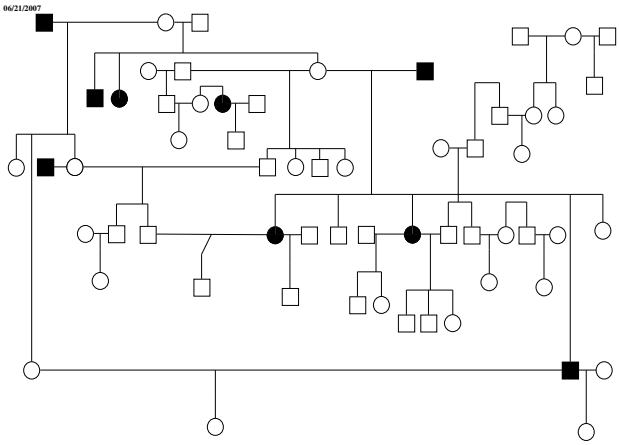

Figure 1. Experimental pedigree for linkage study of DCM in the Great Dane.

Pertinent anthropometric and physiological measures included the internal diameter of the left ventricle at systole and diastole, the percentage of fractional shortening of cardiac cells, and the ejection fraction of blood. Dogs who were diagnosed with DCM typically exhibited 1) an increased internal diameter of the left ventricle during both systole (LVDs) and diastole (LVDd), indicating the thickness of the cardiac muscle, 2) a reduced ejection fraction, the extent to which the amount of blood is pumped from the left ventricle, and 3) a reduced percentage of fractional shortening (FS) relative to normal. FS is a ratio of the difference in the diameter of the left ventricle between the contracted state (systole) and the relaxed state (diastole) (see Table 1), and is used in most canine studies as the major indicator of systolic function[41].

These measurements served as the basis to quantify the aggregate pumping capacity of the heart and whether this ability had been compromised in a manner characteristic of DCM. Normal values for Great Danes were reported by Koch, *et al.* to be as follows: LVDs of 3.95 cm, LVDs of 5.3 cm, and FS of 25%[34]. All diagnoses were made by qualified veterinary cardiologists.

Table 1. Echocardiographic measurements in dogs with (n=6) and without (n=15) DCM. P-values: 1.3x10-6 for LVDs, 6.5x10-7 for LVDd, and 1.9x10-3 for FS

	Great Danes with DCM	**Great Danes without DCM**
LVDs (cm)	5.66 ± 1.04	3.55 ± 1.06
LVDd (cm)	7.30 ± 0.8	5.50 ± 0.54
FS (%)	21.3 ± 7.9	35.4 ± 14.6

LVDs, diastolic left ventricular diameter; LVDd, systolic left ventricular diameter; FS, fractional shortening

Complex Segregation Analysis

iBay v1.1 (Janss, 2006) was used to assess the contribution of a major genetic factor to DCM transmission in the experimental population. As described elsewhere[71], iBay implements a hierarchical Bayesian approach to CSA that harnesses Markov chain Monte Carlo (MCMC) estimation and Gibbs sampling procedures to generate posterior density distributions and Bayesian factors for key model parameters. The approach enables simultaneous evaluation of a putative Mendelian locus and estimation of the posterior density for a polygenic contribution to DCM. Moreover, and in notable contrast to most statistical genetic algorithms available for segregation analysis, iBay supports the use of general pedigrees with multiple generations and inbreeding loops, thus ameliorating the potential loss of power associated with "cutting" kindreds into smaller subsets comprised of "loop-free" nuclear families.

Linkage Study

Whole blood or buccal swabs were collected for each Great Dane in the experimental population. Samples were submitted and DNA was extracted using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN).

For this study, a multiplexed set of canine microsatellite markers, the Minimal Screening Set-2 (MSS-2)[72], was used. The MSS-2 consists of 327 microsatellite markers located throughout the canine genome. It includes 171 tetra-, 151 di-, and three tri-nucleotide repeats that are multiplexed into 69 sets for ease of amplification[73]. The average spacing between MSS-2 markers is nine Mb.

Genotyping of each dog was carried out as described elsewhere[73]. Briefly, MSS-2 primers were used to amplify canine microsatellites by the polymerase chain reaction (PCR). PCR products were resolved on an ABI 3730x1 DNA Analyzer (Applied Biosystems, Foster City, CA). The GeneScan 500 LIZ internal size standard (Applied Biosystems) was used. Genotypes were scored using Genemapper® Software v3.5 (Applied Biosystems).

Two-point LOD scores were calculated with the use of the SOLAR (Sequential Oligogenic Linkage Analysis Routines) software package. A total of three traits were used for statistical analyses, including LVDs, LVDd, and FS. The ejection fraction was not used as a trait, because it is not routinely measured during echocardiography and was not available for all dogs.

Sequencing of SGCG

Three Great Danes with DCM and two without DCM were selected for sequencing. Because DCM is an adult-onset disease, unaffected subjects were selected from the oldest dogs (8 years and 11 years) in our pedigree. In addition, an unaffected mixed-breed dog from a pedigree with no history of cardiac disease was sequenced as a control.

Primers were designed for each of the seven coding exons of *SGCG* in order to amplify the entire exons and to capture the intron-exon boundaries and partial intronic sequences, as well (see Table 2). Sequencing reactions were carried out in a volume of 20µL consisting of 0.25 units/µL Taq DNA polymerase with 1.2X Buffer B (Fisher

Scientific, Pittsburgh, PA), 1.5 mM $MgCl_2$, 1x MasterAmp PCR Enhancer (Epicentre Technologies, Madison, WI), 1 mM dNTPs, 0.5 µM each of forward and reverse primers, and 2.5 ng/µL DNA. All exons were amplified by: 5 min at 95°C followed by 35 cycles of 1 min at 95°C, 30 sec at optimum primer annealing temperature (see Table 2), and 1 min at 72°C, with a final extension of 10 min at 72°C.

Sequences were aligned with the publicly available Boxer sequence using ClustalW (http://www.ebi.ac.uk/clustalw/). It is important to note that, while the Boxer is among the breeds of dog that are afflicted with DCM, the publicly available Boxer sequence was obtained from a healthy Boxer with no evidence of DCM.

Table 2. Canine *SGCG* Primers

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')	Optimum Anneal Temp (°C)	Product Size (bp)
SGCGEx2	TATGGTCACTGGATTTC	ATGATGTGATACAACAAG	45°	635
SGCGEx3	GCATCTGTAATCCC	GAGTGTAACAGCAATG	43°	310
SGCGEx4	GATCTGTGACTATGGAG	CTATTCTCTGTGGTC	43°	358
SGCGEx5	CTGACACTACATATAG	AGTTCTCATCATAAG	38°	348
SGCGEx6	GCTTTATGCCTGCCTTG	GATCTGGTAATGACTGC	49°	497
SGCGEx7	CTGCCATCTGAAACTAC	CATCAGGACATACAAC	45°	435
SGCGEx8	CAACCTGTCTGGAGC	GTGTCTAATAATGTGC	45°	477

Results

Complex Segregation Analysis

The results of CSA do not support transmission of a major genetic locus in this extended pedigree of Great Danes (data not shown). The 95% highest density regions (HDR) for both polygenic and major gene variance were zero, and the Bayes Factor

(logBF) estimated for the fitted mixed model was low enough to be considered "not worth mentioning"[71].

LOD Scores from Linkage Study

All dogs were genotyped at 324 loci, 314 of which are represented in the MSS-2 marker set. Remaining markers from the MSS-2 could not be amplified, or their genotypes were uninformative for linkage. Ten additional markers were added to the set to fine map regions for which positive LOD scores were obtained. The maximum LOD scores are displayed in Table 3, including eight markers on six chromosomes.

Table 3. Markers producing positive LOD scores

Marker Name	Chromosome	LOD Score
FH2132	CFA02	1.6
FH2608	CFA02	1.0
REN162C04	CFA07	1.2
C09.173	CFA09	1.1
FH2885	CFA09	1.1
FH2324	CFA25	1.1
FH2585	CFA28	1.0
RVC11	CFA31	1.1

Sequencing of SGCG

The seven coding exons of *SGCG* were amplified and sequenced, along with portions of the intronic sequences and the intron-exon boundaries. No sequence differences were found between affected and unaffected dogs, in the unaffected mixed-

breed dog, or between the coding sequence of the Great Dane and the publicly available 7X Boxer sequence.

Discussion

DCM is a cardiac disease that results in dilation of the left ventricle of the heart, inhibition of proper blood flow throughout the body, and high mortality of affected dogs[62]. The mode of inheritance and specific causes of the disorder are unknown at present, although one study has suggested that the disorder may be caused by a viral or bacterial agent[62]. In this study, segregation, linkage, and sequencing analyses were conducted in an extended pedigree of Great Danes in an effort to identify genetic factors that play a role in DCM pathogenesis.

CSA can be performed on a pedigree of related individuals in order to evaluate the transmission of a trait of interest from generation to generation[74]. Results from CSA suggest that DCM is not likely to be inherited in a simple Mendelian fashion and might best be interpreted as a complex, multi-factorial disease whose onset and severity is dictated by multiple genetic, environmental, and behavioral factors. That stated, the modest size of the research population must temper conclusions. Also, results for CSA may change if larger, more informative families with more affected individuals were available for future analyses.

Linkage studies of more than 200 markers have failed to detect linkage between DCM and specific chromosomal regions in the Newfoundland[75]. Work here for linkage analysis of 25 related Great Danes was conducted with the hope of identifying candidate

gene regions that might cause or predispose individuals to be affected with DCM. There were positive LOD scores for eight markers located on six different chromosomes. None of the estimated LOD scores met or surpassed the classical threshold of statistical significance (LOD $\geq$ 3.0). However, given the sample size and implemented model specifications, the maximum *possible* LOD appears to be 1.5, perhaps indicating that these results may actually be suggestive of linkage.

Canine chromosome 2 exhibits sequence homology with human chromosomes 1, 5, 10, and 16[16]. Interestingly, the region for which the highest LOD score of 1.6 was obtained has no corresponding conserved segments in the human and has not yet been completely annotated in the dog, preventing further analysis. It is possible that this region is canine-specific and therefore unique to the dog, making it a good candidate for further investigation, since it may harbor new, interesting sequences that play a role in the disease process of DCM.

The six chromosomes listed in Table 3 were further investigated to determine whether any candidate genes were located close to each linked marker. One gene, *SGCG*, consisting of eight exons, the first of which is non-coding, is located approximately 1.6 Mb from FH2324 on CFA25. *SGCG* mutations were previously identified by Noguchi, *et al*. (1995) as causative of the severe childhood autosomal recessive muscular dystrophy (SCARMD), a disease that results in skeletal muscle anomalies and cardiomyopathy of affected patients[44]. In addition, Hack, *et al*. (1998) reported cardiomyopathy development in SGCG-deficient mice[76].

Based on this, the seven coding exons, the intron-exon boundaries, and portions of the intronic sequences of *SGCG* were sequenced. No sequence differences 1) between affected and unaffected Great Danes or 2) between Great Danes in the experimental population and the publicly available 7X Boxer sequence were found. These findings appear to exclude sequence variation within the coding regions and intron-exon border regions of *SGGG* as the cause of DCM in the Great Dane.

This study did not evaluate non-coding or regulatory regions of *SGCG*, or the potential contribution of aberrations of protein structure and function. Certainly it would be advantageous to verify the presence of the functional protein product of *SGCG* through western blotting or immunohistochemical staining. In addition, mRNA levels could be determined for both affected and unaffected dogs in order to determine if transcripts are produced. Promoter regions and binding sites for transcription factors could be evaluated for mutations that alter promoter function.

Recently, an array containing over 50,000 SNPs has become available through Affymetrix, enabling genome wide comparisons of SNPs between different DNA samples. Evaluating such a large number of SNPs in DNA from affected and unaffected dogs would greatly increase the likelihood of finding linkage to causative or susceptibility genes. One drawback of this approach, however, is the requirement of a large number of affected dogs. For complex diseases such as DCM, a minimum of 100 affected and 100 unaffected dogs is required. However, if such a sample size were feasible, SNP analysis would be an excellent option.

Genetic analysis of inherited diseases is complicated when there is no simple pattern of inheritance. In fact, complex traits rarely follow simple Mendelian inheritance, due to the possibility of many contributing genes or loci and interactions with environmental factors. These contributing loci, also called quantitative trait loci (QTLs), are not easily mapped with simple mapping, due to their distribution throughout the genome. QTL mapping typically assumes equal contribution of all loci involved in the trait. Since this cannot be accurately assessed for inherited diseases that have genetic and environmental factors, it is not always feasible. Further, QTL mapping is an approach reliant on phenotypic characterization. Since DCM is an adult-onset disease, phenotypic characterization is often unreliable for animals that are younger than the typical age of onset.

Conclusions

This work has identified eight chromosomal regions that may influence DCM risk in Great Danes. Continued evolution and expansion of the canine genome map will no doubt lead to expanded linkage marker sets, and use of single nucleotide polymorphism array technology will accelerate identification of loci that are associated with various hereditary diseases. Future studies will focus on the acquisition of additional families, collection of more expansive clinical and genotype data, and use of SNP arrays to dissect the complex nature of canine DCM.

CHAPTER III

CONCLUSIONS

Studies of the domestic dog have revealed causative genes for many hereditary diseases. Because of the similarity of the genomes of the dog and human, it is possible to utilize information discovered through studies of canine hereditary diseases and apply them to studies of hereditary diseases that afflict humans. DCM causes death in both the dog and human, and an estimated 30% of cases of DCM are thought to be familial. Determination of the mode of inheritance of DCM in the Great Dane would enable breeders to identify individuals that should be eliminated from breeding. In addition, linkage approaches in the dog can help localize the search for genes, as well, by revealing regions in the genome that are coinherited with alleles causing a disease.

Chapter II provides the results of a linkage study in a family of Great Danes in order to identify regions of the Great Dane genome that are linked to DCM. This work revealed linkage of DCM to six different chromosomes (chromosomes 2, 7, 9, 25, 28, and 31). However, canine chromosome 2 warrants further investigation, because it contains a region which has no corresponding conserved segments in the human. Sequencing of one candidate gene, *SGCG*, was performed in order to determine its relationship to DCM. Unfortunately, no differences in sequences between affected and unaffected Great Danes were found. Furthermore, the sequence from Great Danes was compared to the Boxer reference sequence and revealed no differences. This work excluded the coding portions of *SGCG* as causative for DCM in Great Danes.

In summary, the domestic dog serves as a valuable model for human disease. The dog has historically been valued by humans for many reasons, not the least of which being a faithful companion. This work has evaluated DCM which causes significant mortality in many breeds as well as the human. Understanding DCM in the Great Dane may enhance our understanding of human DCM, but will undoubtedly also improve the lives of countless Great Danes.

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VITA

Name: Stephanie Michelle Herbst

Permanent Address: VMS Bldg, Rm 203
Texas A&M University
College Station, Texas
77843-4467

Education: Texas A&M University
College Station, Texas
B.S., 2000, Cell and Molecular Biology
B.S., 2002, Genetics

Texas A&M University
College Station, Texas
Ph.D., 2007, Genetics